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Effective silencing of ENaC by siRNA delivered with epithelial-targeted nanocomplexes in human cystic fibrosis cells and in mouse lung

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Complete List of Authors:	<p>Tagalakis, Aristides; University College London, UCL Great Ormond Street Institute of Child Health</p> <p>Munye, Mustafa; University College London, UCL Great Ormond Street Institute of Child Health</p> <p>Ivanova, Rositsa; University College London, Neuroscience, Physiology and Pharmacology</p> <p>Chen, Hanpeng; King's College London, 3Institute of Pharmaceutical Science</p> <p>Smith, Claire; University College London, UCL Great Ormond Street Institute of Child Health</p> <p>Aldossary, Ahmad; University College London, UCL Great Ormond Street Institute of Child Health</p> <p>Rosa, Luca; University College London, UCL Great Ormond Street Institute of Child Health</p> <p>Moulding, Dale; University College London, UCL Great Ormond Street Institute of Child Health</p> <p>Barnes, Josephine; University College London, UCL Respiratory Centre for Inflammation and Tissue Repair</p> <p>Kafetis, Konstantinos; University College London, UCL Great Ormond Street Institute of Child Health</p> <p>Jones, Stuart; King's College London, Institute of Pharmaceutical Science</p> <p>Baines, Deborah; St George's, University of London, Biomedical Sciences</p> <p>Moss, Guy; University College London, Neuroscience, Physiology and Pharmacology</p> <p>O'Callaghan, Christopher; University College London, Respiratory, Critical Care & Anaesthesia</p> <p>McAnulty, Robin; UCL, Medicine</p> <p>Hart, Stepehn; University College London, UCL Great Ormond Street Institute of Child Health</p>
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Effective silencing of ENaC by siRNA delivered with epithelial-targeted nanocomplexes in human cystic fibrosis cells and in mouse lung

Aristides D. Tagalakakis¹, Mustafa M. Munye¹, Rositsa Ivanova², Hanpeng Chen³, Claire M. Smith⁴, Ahmad M. Aldossary¹, Luca Z. Rosa¹, Dale Moulding⁵, Josephine L. Barnes⁶, Konstantinos N. Kafetzis¹, Stuart A. Jones³, Deborah L. Baines⁷, Guy W. J. Moss², Christopher O'Callaghan⁴, Robin J. McAnulty⁶ and Stephen L. Hart¹

¹Experimental and Personalised Medicine Section, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK

²Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street, London, WC1E 6BT, UK

³Institute of Pharmaceutical Science, Faculty of Life Science & Medicine, King's College London, 150 Stamford Street, London, SE1 9NH, UK

⁴Respiratory, Critical Care & Anaesthesia, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK

⁵UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK

⁶UCL Respiratory Centre for Inflammation and Tissue Repair, 5 University Street, London, WC1E 6JF, UK

⁷Institute of Infection and Immunity, St George's University of London, London, SW17 0RE, UK

Running title: Silencing of ENaC for CF treatment

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Correspondence to

Dr Aristides D Tagalakakis, Experimental and Personalised Medicine Section, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK; a.tagalakakis@ucl.ac.uk

ABSTRACT

Introduction Loss of the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis (CF) leads to hyperabsorption of sodium and fluid from the airway due to upregulation of the epithelial sodium channel (ENaC). Thickened mucus and depleted airway surface liquid (ASL) then lead to impaired mucociliary clearance. ENaC regulation is thus a promising target for CF therapy. Our aim was to develop siRNA nanocomplexes that mediate effective silencing of airway epithelial ENaC *in vitro* and *in vivo* with functional correction of epithelial ion and fluid transport.

Methods We investigated translocation of nanocomplexes through mucus and their transfection efficiency in primary CF epithelial cells grown at air-liquid interface (ALI). SiRNA-mediated silencing was examined by quantitative RT-PCR and Western analysis of ENaC. Transepithelial potential (V_t), short circuit current (I_{sc}), ASL depth and ciliary beat frequency (CBF) were measured for functional analysis. Inflammation was analysed by histological analysis of normal mouse lung tissue sections.

Results Nanocomplexes translocated more rapidly than siRNA alone through mucus. Transfections of primary CF epithelial cells with nanocomplexes targeting α ENaC siRNA, reduced α ENaC and β ENaC mRNA by 30%. Transfections reduced V_t , the amiloride-sensitive I_{sc} , and mucus protein concentration, while increasing ASL depth and CBF to normal levels. A single dose of siRNA in mouse lung silenced ENaC by approximately 30%, which persisted for at least 7 days. Three doses of siRNA increased silencing to approximately 50%.

Conclusion Nanoparticle mediated-delivery of ENaC siRNA to ALI cultures corrected aspects of the mucociliary defect in human CF cells and offers effective delivery and silencing *in vivo*.

Keywords: ALI, nanoparticle, ENaC, cilia, ASL, cystic fibrosis, airway epithelia

What is the key question?

Can silencing of the airway epithelial sodium channel (ENaC) activity correct the mucociliary defects associated with the CF epithelium?

What is the bottom line?

SiRNA-mediated silencing of ENaC *in vitro* in pseudostratified, ciliated, air-liquid interface (ALI) models of the human airway was shown to correct the electrical and mucociliary defects associated with the CF epithelium and, in addition, we demonstrated efficiency of delivery *in vivo* to murine lung and accumulation of the level of silencing by repeated delivery along with safety by analysis of inflammation.

Why read on?

We have described a siRNA nanoparticle formulation that penetrates mucus to transfect CF epithelial cells at ALI and following repeated transfection both at ALI and *in vivo* demonstrated cumulative ENaC silencing to achieve the desired functional effect, providing evidence to support its further development as a novel therapeutic approach.

INTRODUCTION

Cystic Fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) which encodes a cyclic AMP-activated channel for chloride and other anions.¹⁻² Mutations in CFTR also result in upregulation of the epithelial sodium channel, ENaC, leading to imbalanced water and ion movement across the airway epithelium.³⁻⁴ This results in depletion of the airway surface liquid (ASL) and thickened mucus with progressive loss of pulmonary function.³⁻⁴ Therefore, ENaC is a promising therapeutic target for CF with the potential to restore lung fluid homeostasis and thus improve mucociliary clearance. ENaC is comprised of α , β and γ subunits. The pore forming α ENaC subunit is required for full channel function. The β and γ subunits are regulators of ENaC activity⁵⁻⁶ and residual ENaC activity can be measured in their absence.^{5, 7} The other ENaC subunit, δ , is only expressed at low levels in the lung of humans and not at all in rodents.⁸

Small molecule ENaC inhibitors such as amiloride⁹ and benzamil¹⁰ reduce sodium uptake but their effects are short-lived because of drug absorption.¹¹ Silencing of ENaC expression, by short interfering RNA (siRNA)-mediated RNA interference (RNAi), offers a more promising therapeutic route.¹²⁻¹⁴ Potential advantages of nanoparticle-mediated siRNA therapy include its potency, specificity, duration,¹⁵ and restriction to the airways to prevent nephrotoxicity. Transfection of the CF airway epithelium requires protection of siRNA from nucleases, penetration of the mucus and periciliary liquid layer (PCL) and targeted uptake by the epithelial cells¹⁶ with purpose-designed, nanoparticles. Previous attempts to develop ENaC siRNA therapies¹⁷⁻¹⁸ were limited by lack of nanoparticles capable to effectively deliver siRNA to the airway epithelium.

Receptor-targeted nanocomplexes (RTNs) comprise multi-functional mixtures of cationic

liposomes (L) and cationic targeting peptides (P) which self-assemble, electrostatically on mixing with siRNA (R).¹⁹⁻²⁴ Peptide E packages nucleic acids through a cationic, oligolysine domain and mediates epithelial receptor targeting through a seven amino acid motif, SERSMNF, derived by biopanning of a phage peptide library.²⁵ The targeting peptide displays close similarity to receptor binding proteins of two intracellular pathogens, rhinovirus and *Listeria monocytogenes*.²⁶ Rhinoviruses bind intercellular adhesion molecule-1 (ICAM-1), that is present in the airway epithelium, and upregulated in the inflamed CF epithelium.²⁷⁻²⁸ Following receptor-mediated endocytosis the liposome component destabilises the endosomal bilayer allowing nucleic acid release to the cytoplasm before endosomal degradation occurs.²⁹ This peptide has been used previously for targeted transfection with plasmid DNA,²⁵ minicircle DNA³⁰ and siRNA³¹ of bronchial epithelial cells *in vitro* and *in vivo*.

In this study, we have investigated the effects of RTN-mediated delivery of α ENaC siRNA on pseudostratified, ciliated, air-liquid interface (ALI) models of the human CF airway, particularly the correction of the electrical and mucociliary defects associated with the CF epithelium. Finally, we have investigated the translational potential of this therapy by delivery of murine α ENaC siRNA to the lungs of normal mice to assess *in vivo* transfection efficacy in a surrogate model of the human lung, including efficacy of repeated delivery as well as safety by analysis of inflammation.

MATERIALS AND METHODS

Full details are available in the online supplementary material.

Statistics

Data are expressed as the mean \pm standard error of the mean (SEM) and analysed using a two-tailed, unpaired Student's *t*-test or one-way analysis of variance (ANOVA) and Bonferroni's post hoc analysis where applicable. We also report other data as median and interquartile range (IQR) and these have been analysed using a Mann-Whitney *U* non-parametric test.

RESULTS

Assessment of nanocomplex translocation through mucus

Nanocomplexes containing Cy3-labelled siRNA were added to the surface of the mucus, then the cumulative concentration of fluorophores diffusing through the mucus into the lower collection chamber over 60 minutes was measured and compared to siRNA alone and fluorescent cationic, polystyrene nanoparticles. The concentration of RTNs penetrating through the CF human airway mucus barrier at 1 h (figure 1C and Table S1) ($441.1 \pm 52.7 \text{ ng/cm}^2$) was 46% of that collected from the normal human airway mucus barrier at 1 h ($961.5 \pm 44.8 \text{ ng/cm}^2$; figure 1B) and this was similar for siRNA alone at 43% ($390.1 \pm 15.8 \text{ ng/cm}^2$ through CF human airway mucus and $917.3 \pm 60.1 \text{ ng/cm}^2$ through normal human airway mucus). RTN and siRNA penetration through normal mucus were similar at 1 h (figure 1B) ($p>0.05$, $n=6$), whereas in porcine gastric mucus the RTNs penetrated more rapidly than siRNA (figure 1A) ($1046.1 \pm 47.6 \text{ ng/cm}^2$ and $472.0 \pm 8.8 \text{ ng/cm}^2$ for RTNs and siRNA alone, respectively; $p<0.01$, $n=6$). The rate of penetration of pig gastric mucus by cationic polystyrene (PS) nanoparticles ($55.0 \pm 2.1 \text{ nm}$ and $+23.1 \pm 1.1 \text{ mV}$) was not significantly different to siRNA alone (figure 1A; $782.7 \pm 123.0 \text{ ng/cm}^2$ and $472.0 \pm 8.8 \text{ ng/cm}^2$ for PS nanoparticles and siRNA alone, respectively at 1 h) but in CF mucus, PS nanoparticles were significantly slower than RTNs or siRNA (figure 1C; $100.0 \pm 57.6 \text{ ng/cm}^2$ for PS nanoparticles at 1 h compared to the concentrations displayed above which were ~4-fold more for both RTNs and siRNA alone; $p<0.05$, $n=6$).

Diffusion coefficients in the three types of mucus (D_m) and water (D_w) were calculated as described in the Methods section. D_m/D_w is the relative restriction of diffusion in mucus compared to water (Table 1). The diffusion of RTNs was 14-fold higher than that of siRNA alone in CF mucus (27-fold impedance for RTNs compared to 376-fold impedance for siRNA

alone) despite the nanoparticles being significantly larger (91.9 ± 0.5 nm) and more cationic ($+35.1 \pm 0.6$ mV) than molecular siRNA which is approximately 7.5 nm.³² RTNs penetrated normal human mucus and pig gastric mucus 8.2 and 12.3-fold faster, respectively, than siRNA alone (Table 1).

***In vitro* siRNA silencing of ENaC in epithelial cells**

Peptide-targeted cationic nanocomplexes were then used to transfect 16HBE14o- epithelial cells with siRNA targeting α ENaC. Western blot analysis of α ENaC protein showed that transfection of 16HBE14o- cells with RTNs at 75 nM siRNA led to a decrease in the abundance of both the 90 kDa and the 65 kDa α ENaC protein bands by 41% and 48%, respectively compared to those transfected with control siRNA (figures S1A, B).

Expression of α ENaC in *BMI-1* transduced CF bronchial epithelial cells³³ (CFBE) attained maximal levels after 2-5 days in ALI cultures (figure 2A) and so, ALI culture transfections in ongoing experiments were performed after at least 5 days ALI culture. Transfections of CFBE monolayers with 100 nM α ENaC siRNA, reduced α ENaC mRNA by 30% (n=3) compared to control siRNA-treated cultures (figure 2B). After three sequential transfections, performed at 48 h intervals, the level of silencing was improved to 54% (n=3; figure 2B). Silencing of α ENaC also resulted in a 51% silencing of β ENaC (n=3; Table S2) but not the γ subunit (figure 2C). The α subunit in CFBE cells was 74.9 and 19.7-fold overexpressed relative to the γ and β subunits, respectively (figure S2, n=3).

Functional effects of ENaC silencing

We next investigated the effects of α ENaC silencing on amiloride-sensitive ENaC-mediated

short circuit current (I_{sc}) in CFBE cells cultured at ALI two days after transfection (Figure 3A). The amiloride-sensitive I_{sc} was reduced in cells treated with α ENaC siRNA (median: 6.4 μ A/cm²; IQR: 5.4-9.8 μ A/cm²; n=6) compared to control siRNA (median: 11.5 μ A/cm²; IQR: 10.1-14.1 μ A/cm²; $p<0.05$, n=8) or untreated cells (median: 14.3 μ A/cm²; IQR: 13.2-17.9 μ A/cm²; $p<0.01$, n=5) (figures 3B and 3C; Table S3). As expected, CFBE cells showed a very limited response to forskolin (a cAMP agonist) or GlyH-101 (a CFTR inhibitor) under any of the conditions examined (figure 3B) as opposed to NHBE cells (figure S3). Transepithelial electrical resistance (R_t) was not perturbed by the transfection procedure (figure S4), with ENaC-silenced cells having a median of 693.5 (IQR: 565.7-805.8) Ω cm² (n=7) compared to 783.2 (IQR: 711.1-953.2) Ω cm² and 676.7 (IQR: 653.1-744.2) Ω cm² for control siRNA (n=7) and untreated cells (n=3), respectively (Table S3).

We then performed three sequential siRNA transfections of CFBE cells at 48 h intervals and determined physiological responses to ENaC silencing. Mucus was not removed during this period (unwashed cells). Samples treated with α ENaC siRNA had lower negative transepithelial potentials (V_t) (median: -7.2 mV; IQR: -5.7 to -12.2 mV), than those transfected with control siRNA (median: -16.0 mV; IQR: -14.8 to -19.0 mV) (figure 3D; n=5) as measured by Scanning Ion Conductance Microscopy (SICM). The median V_t value for the VX-770 and VX-809-treated CF cells was -6.8 mV (IQR: -6.7 to -12.7 mV) while for untreated, non-CF cells it was -7.7 mV (IQR: -6.3 to -11.6 mV; Table S4).

The ASL depth in CFBE ALI cultures was increased by ~1.5-fold (median: 12.1 μ m ; IQR: 10.7-14.9 μ m) after silencing of α ENaC compared to control siRNA-treated (median: 7.9 μ m; IQR: 6.4-9.8 μ m) and untreated cells (median: 8.2 μ m; IQR: 5.8-11.1 μ m) ($p<0.001$, n=4) (figures 4A and 4B; Table S4). The ciliary beat frequency (CBF) of unwashed CFBE cells

increased to 14.5 ± 0.5 Hz from 9.6 ± 0.7 Hz in untreated CFBE cultures following sequential treatment with α ENaC siRNA ($p < 0.001$, $n=10$), compared to 11.9 ± 1.0 Hz in control siRNA-treated cells ($p < 0.05$, $n=10$) (figure 4C). The CBF of VX-770 and VX-809-treated cells was also increased to 12.8 ± 0.5 Hz and ($p < 0.05$, $n=10$; Table S4).

Cells treated with α ENaC siRNA displayed reduced net fluid absorption rates from the apical side of the epithelium with a median value of $0.9 \mu\text{l}/\text{cm}^2/\text{h}$ (IQR: $0.6\text{-}1.3 \mu\text{l}/\text{cm}^2/\text{h}$) which was lower than that of both control siRNA-treated cells (median: 1.6 ; IQR: $1.3\text{-}1.9 \mu\text{l}/\text{cm}^2/\text{h}$; $p < 0.05$, $n=4$) and untreated cells at $1.5 \mu\text{l}/\text{cm}^2/\text{h}$ (IQR: $1.1\text{-}1.9 \mu\text{l}/\text{cm}^2/\text{h}$; figure 4D; Table S4). Finally, we measured the total protein concentration in mucus collected from each well of the sequentially-transfected CF monolayers. The apical surface of each well was washed with PBS 2 days after the first (figure 5A) and second transfections (figure 5B) and 7 days after the third (figure 5C) transfection. In all cases, the protein concentration in mucus from the α ENaC-silenced cells ($n=6$) was significantly lower than that from the control siRNA-treated cells ($n=6$), VX-770 and VX-809-treated cells ($n=4$) or untreated cells ($n=3$) and this effect lasted for at least 7 days post-transfection. At the end of the experiment, at 8 days post-transfection with α ENaC siRNA ($n=6$), α ENaC expression was still 27% lower than control siRNA-treated cells ($p < 0.05$; figure 5D).

In vivo lung delivery

We then investigated delivery of siRNA into the lungs of normal mice to assess the translational potential of α ENaC siRNA delivery. The biodistribution analysis of nanocomplexes containing Dy677-labelled siRNA 24 h after oropharyngeal instillation showed very high retention of siRNA in lungs (figure 6A) with low-level fluorescence in intestines (figure S5, $p < 0.05$, $n=3$)

which probably reflects incidental swallowing during administration. There was no fluorescence in heart, liver, kidneys and spleen suggesting no detectable transfer of the siRNA from the lung to the circulation (figure 6A). Transfecting mice by oropharyngeal instillation with α ENaC siRNA (n=7) or control siRNA (n=7), silenced α ENaC by 30% at the mRNA level compared to control siRNA ($p<0.01$) (figure 6B). Silencing of α ENaC 1 week after transfection remained at 23% compared to control siRNA (figure 6C; n=4 for untreated, n=6 for control siRNA and n=7 for α ENaC siRNA-treated mice) and was not significantly different from silencing at 48 h. Repeated transfections with α ENaC siRNA (n=8) demonstrated cumulative silencing, increasing to 58% reduction compared to control siRNA at 72 h after the third instillation of α ENaC siRNA (figure 6D; Table S5).

The RTN siRNA formulations were well tolerated by the mice for single or triple dosing (figures S6A, B). H&E staining of lung sections (n=3) showed that treatment with RTNs containing ENaC siRNA induced sporadic mild peribronchial cell infiltrates, the size and severity of which was unaffected by the number of instillations (figure 7A-B). Control siRNA induced a similar inflammatory response although the foci were generally smaller and less frequent than with ENaC siRNA (figure 7C).

DISCUSSION

The lack of functional CFTR and hyperactivity of ENaC in the airways of CF patients leads to disrupted ion and fluid homeostasis.³ Modulators of CFTR such as Ivacaftor and the Ivacaftor/Lumacaftor (Orkambi) combination therapy offer treatment for patients with specific mutations³⁴⁻³⁵ but there are some mutation classes where CFTR modulators will not be effective, such as nonsense mutations, and so other therapeutic strategies are required. The low-volume hypothesis of CF lung disease links increased activity of ENaC with ASL depletion in CF³ and so reducing transepithelial sodium absorption through ENaC is an alternative target for therapeutics.^{11,36} The role of ENaC in regulating ASL volume has been validated in recent work showing that inhibition of ENaC proteolytic activation using the SPLUNC1 or peptide fragments³⁷⁻³⁹ or QUB-TL1⁴⁰ successfully prevented dehydration of the ASL in CF HBEC monolayers. Small molecule inhibitors of ENaC suffer from rapid systemic absorption from the lung and are associated with side effects such as hyperkalaemia due to ENaC inhibition in the kidney or pulmonary oedema.^{11, 41-42} We have therefore investigated ENaC silencing mediated by nanoparticles carrying siRNA.

We used siRNA to target the major subunit, α ENaC. The α ENaC subunit forms a sodium-conducting pore, while the other two subunits enhance its activity.⁴³ The α subunit is critical for sodium transport function and volume regulation as demonstrated in α ENaC knockout mice, which die soon after birth due to a failure to clear their lungs of fluid.⁴⁴ In addition, it has been shown that a low mRNA abundance of α ENaC in the nasal epithelium of premature infants is linked with respiratory failure.⁴⁵ Lastly, a mutation that leads to α ENaC hyperactivity was found in patients with atypical CF.⁴⁶

Nanoparticles offer protection to the siRNA during nebulisation and from nuclease attack

in lung fluids, and enable penetration of extracellular barriers such as mucus and the PCL⁴⁷ before entering the epithelial cells by endocytosis. RTNs, comprising the oligolysine epithelial-targeting peptide (Peptide E) and the liposome DOTMA/DOPE, self-assemble on mixing lipid and peptide components with siRNA to form cationic, monodisperse nanoparticles with a size of approximately 90 nm, which have been used previously for lung delivery of nucleic acids (including by nebulisation) in mice and pigs.^{25-26, 30-31, 48} Similar RTN formulations can package and transfect siRNA efficiently^{20, 23-24, 49} and so we are now investigating use of α ENaC siRNA to assess its therapeutic potential in epithelial lung models *in vitro* and *in vivo*.

We first assessed the ability of RTNs to penetrate mucus, which presents a physical barrier to nanoparticle siRNA delivery in the airways. Mucus is a gel-like layer, rich in charged mucin glycoproteins covering the lung epithelium and is particularly thick and sticky in the CF lung.⁵⁰⁻⁵¹ Differentiated ALI cultures of human airway epithelial cells produce copious amounts of mucus, which is particularly viscous from CF cells.⁵² RTN diffusion in mucus from three sources was used including porcine gastric mucus, and human mucus from differentiated CF and non-CF airway epithelial cultures. RTN diffusion rates were lowest in CF mucus, consistent with its increased viscosity. RTNs diffused at similar rates to siRNA alone in CF and non-CF mucus despite their size and charge differences. The diffusion rates of RTNs were greater than those of cationic polystyrene nanoparticles of similar size used in cervicovaginal mucus,⁵³⁻⁵⁴ suggesting that surface properties of RTNs contribute to their mucus mobility.³²

Silencing levels of α ENaC by 30% from a single dose were achieved, which resulted in a significant reduction in amiloride-sensitive I_{sc} compared with control siRNA and untransfected cells. Silencing did not affect R_t indicating that there was no damage to the epithelial tight junctions by the nanoparticle complex directly or by decreased cell viability and that the changes

in I_{sc} were a direct effect of reduced α ENaC activity. Interestingly β ENaC, but not γ ENaC, was also silenced by α ENaC siRNA. This was observed in other studies,¹⁷ suggesting transcriptional feedback inhibition of β ENaC by α ENaC.

Enhancement of silencing of α ENaC to 50% by three sequential transfections in CFBE cells reduced the transepithelial potential to normal range values (-7 mV) in ALI cultures.⁵⁵ This may be a useful therapeutic biomarker as CF individuals display hyperpolarised nasal transepithelial potentials.⁵⁶ Silencing of α ENaC also caused a significant reduction in fluid absorption and increased ASL depth to normal levels. Increased hydration of the epithelial surface was also indicated by the reduced total protein concentration in mucus washings from α ENaC siRNA-treated cultures for at least a week post-transfection whereas the CFTR potentiator/corrector drug combination, VX-770 and VX-809, showed no effect on mucus protein concentration.

We further hypothesised that increased ASL depth would reduce impedance of ciliary motility. Indeed we found that the ciliary beat frequency (CBF) in ENaC siRNA-treated CF cells was increased to ~15 Hz which compares well with normal CBF of approximately 16 Hz.⁵⁷ Furthermore, the siRNA induced changes in CBF and fluid height after 3 doses, which was consistent with that reported for current drugs used to correct CFTR activity. VX-770 on its own increased CBF,⁵⁸ whereas the VX-770 and VX-809 combination increased ASL height⁵⁹ (both studies following 5 consecutive day treatments).

This study suggests that silencing ENaC in the range of 30-50% is sufficient for restoration of epithelial ion transport balance, fluid transport and mucociliary properties. In previous α ENaC silencing studies with siRNA, transfections were performed pre-ALI as transfections at ALI were ineffective,¹⁸ although they reported correction of the short circuit

current and increased ASL when using siRNAs targeting both α and β ENaC.¹⁸ Another study showed that silencing of α ENaC with a commercial transfection reagent in submerged cultures lasted for at least 3 days although they did not perform transfections at ALI.¹³ They also reported 35-40% silencing *in vivo* with a liposomal formulation from a single administration which persisted at least 72 h.¹³ Finally, an shRNA approach following lentiviral transduction of immortalized and primary cell lines used α ENaC as their target.¹² The authors transduced primary cells before the formation of tight junctions, unlike our fully differentiated cell approach, and showed up to 60% silencing. Similar to our findings they have shown that reducing mRNA results in proportional changes in the short circuit current responses and also reduced net apical to basal fluid flux. In addition, they demonstrated that, at a high lentiviral dose, the β ENaC mRNA was reduced but γ ENaC was not which is in agreement with our findings.

We finally quantified ENaC silencing, after single or repeat siRNA dosing (by oropharyngeal instillation) in the lungs of normal mice. The tolerance of repeated dosing supports the hypothesis that our nanoparticles do not induce neutralising antibodies which is important for a potential life-long therapy. Minimal effective modulation of ENaC activity by repeated dosing of siRNA is likely to be much safer than complete inactivation of α ENaC since this could result in negative effects such as oedema observed in lungs of ENaC knockout mice.⁴⁴ There were no significant adverse effects of repeated dosing of RTNs in mice as judged by body weight and behaviour. There was only minimal focal inflammation assessed histologically and the nanocomplex biodistribution was restricted to the lungs. Transfection experiments at ALI with human CFBE cells suggested that silencing of α ENaC in the range of 30-50% was sufficient for restoration of epithelial ion transport balance, fluid transport and mucociliary properties. We

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3 have shown that RTNs achieve these levels of silencing in mice and that the silencing is
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5 persistent for at least one week. These findings support the translational potential of this
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7 nanoparticle-mediated siRNA therapy for CF.
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10 In summary, we have described nanoparticles that can penetrate mucus effectively and
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12 deliver siRNA to the airway epithelium and that silencing of α ENaC can be achieved *in vitro* and
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14 *in vivo* leading to improved hydration and mucociliary function in CFBE monolayers. Other
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16 studies have also shown functional correction of airway epithelial cells but only by transfection
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18 prior to ALI culture. RTNs represent a powerful new tool for siRNA transfection studies in
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20 differentiated respiratory epithelial cells and *in vivo*. The potential to regulate ENaC to a
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22 minimally effective level, the persistence of silencing for at least one week and restriction of
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24 distribution to the lung after airway administration indicates translational potential and suggests
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26 advantages in efficacy and safety over orally-administered, small molecule drugs.
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Figure Legends

Figure 1 Nanoparticles translocate vertically through the mucus barrier. Cy3-siRNA alone nanocomplexes containing Cy3-siRNA or fluorescent polystyrene nanoparticles (PS) were investigated for their translocation potential through (A) pig gastric mucus, (B) normal human airway mucus and (C) CF human airway mucus. The siRNA that is labelled with Cy3 is targeting GAPDH. The experiments were repeated on three occasions and each point is the mean \pm SEM of triplicate measurements.

Figure 2 ENaC expression over time of primary cells growing at ALI and silencing of ENaC. (A) The fold-difference in endogenous α ENaC gene expression relative to β -actin was quantified by qRT-PCR in CFBE cells from the start date of ALI cultures (mean of $n=3$ per time point). Points represent mean values \pm SEM. (B) RTN formulations containing either α ENaC siRNA or control siRNA at 100 nM were used in transfections of CFBE cells grown at ALI for 6 weeks. Transfections were performed once (single) or sequentially every other day (triple) and the percentage of silencing was calculated 48 h after the last transfection ($n=3$ per formulation). The middle horizontal lines represent the median values. Silencing is normalised to the mean control siRNA set at 100%. (C) CFBE cells grown at ALI were transfected with RTN formulations containing either α ENaC siRNA or control siRNA at 100 nM and the percentage of silencing of α , β and γ ENaC subunits was then calculated 48 h after transfection ($n=3$ per formulation). The middle horizontal lines represent the median values. Silencing is normalised to the mean control siRNA set at 100%. Mann-Whitney U non-parametric tests were performed and no statistical significant differences were achieved.

Figure 3 Single transfection of CFBE air-liquid interface cell monolayers with nanocomplexes containing α ENaC siRNA reduces the amiloride-sensitive short circuit current (I_{sc}). **(A)** A schematic highlighting experiments performed after single or triple transfections on CFBE cells grown at ALI. **(B)** Representative I_{sc} traces from CFBE monolayers in Ussing chambers of cells treated with 100 nM α ENaC siRNA or control siRNA or untreated cells. **(C)** The change in I_{sc} (ΔI_{sc}) after application of 10 μ M amiloride is shown for the α ENaC siRNA-treated cells (Ussing chambers; n=6), the control siRNA-treated cells (n=8) and the untreated CFBE cells (n=5). Median values are presented as bars and IQR by upper and lower horizontal lines, with statistical significance determined by the Mann-Whitney U non-parametric test. Asterisks indicate comparisons of specific formulations with statistical significance (*, $p<0.05$; **, $p<0.01$). **(D)** Transepithelial potential (V_t) of α ENaC siRNA-treated monolayers of CFBE cells cultured for 4 weeks on ALI. Wells were triple transfected with 100 nM α ENaC siRNA (n=5) or control siRNA (n=5) and then V_t measurements performed 3 days after the last transfection using Scanning Ion Conductance Microscopy (SICM). Further samples were treated with 10 μ M VX-809 1 day prior to V_t measurement, and treated with 10 μ M VX-770 during measurement (for approximately 20 minutes; n=4). Median values are presented as bars and IQR by upper and lower horizontal lines. Mann-Whitney U non-parametric tests were performed and no statistical significant differences were achieved.

Figure 4 Effect of triple transfections of CFBE ALI cell with nanocomplexes containing α ENaC siRNA. The CFBE cells were cultured in snapwells for 4 weeks then treated with 100 nM α ENaC siRNA or control siRNA at 48 h intervals and the measurements performed 3 days after

the third dose. **(A)** ASL depth measurements of CFBE monolayers as determined by confocal microscopy. XZ representative images of fluorescently-labelled ASL (red) and cells (green). Top: untreated cells, middle: cells transfected 3 times with 100 nM of control siRNA and bottom: cells transfected 3 times with 100 nM of α ENaC siRNA. A white bar has been included to denote the ASL measurement. **(B)** ASL depth measurement of each treatment group (n=4). Bars represent mean \pm SEM, while asterisks indicate statistical significance of comparisons between the α ENaC siRNA-treated cells versus the controls (***, $p < 0.001$) determined by determined by an ANOVA test followed by Bonferroni's post-hoc test. **(C)** Effect of α ENaC siRNA, control siRNA and VX-770 and VX-809 on ciliary beat frequency (CBF) of CFBE cells grown at ALI. For each experimental condition (n=10), readings of CBF were calculated from ten ciliated areas in the snapwell and the data represent the mean \pm SEM. Asterisks indicate comparisons of specific formulations with statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) determined by an ANOVA test followed by Bonferroni's post-hoc test. **(D)** Transepithelial fluid transport through human CFBE cells. Net fluid absorption rate was measured 4 days after cell treatment with a control siRNA or with α ENaC siRNA. Bars represent the median value with IQR shown by the horizontal lines (n=4), while asterisks indicate statistical significance (*, $p < 0.05$) determined by Mann-Whitney U non-parametric tests.

Figure 5 Protein concentration in mucus collected at different time points from transfected or untreated CFBE cells grown at ALI and *in vitro* silencing after 3 transfections. Mucus collections were done **(A)** 2 days after the first transfection (α ENaC siRNA-treated, n=6; control siRNA-treated, n=3; untreated controls, n=3), **(B)** 2 days after the second transfection (α ENaC siRNA-treated, n=6; control siRNA-treated, n=3; VX-770 and VX-809-treated, n=3; untreated controls,

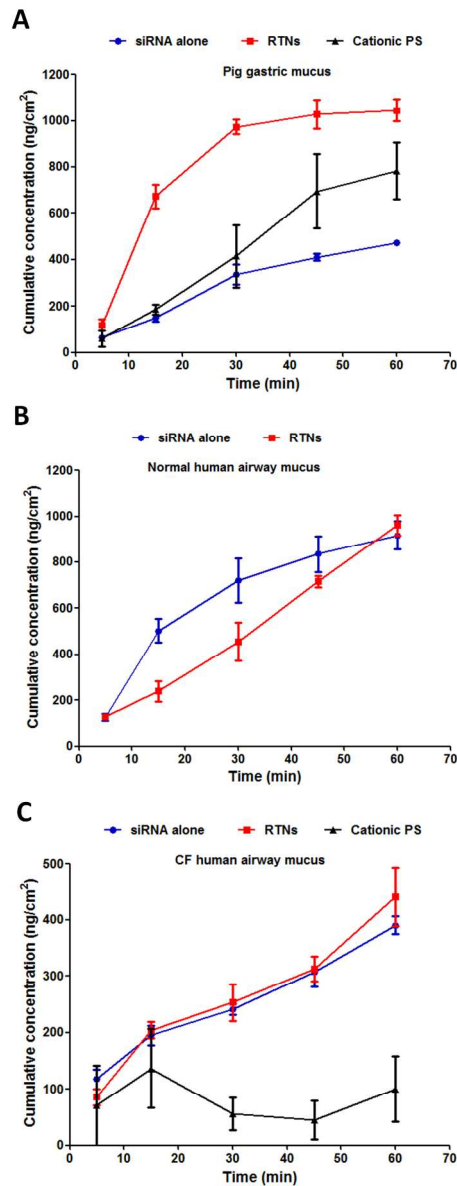
n=3) and **(C)** 7 days after the third transfection (α ENaC siRNA-treated, n=6; control siRNA-treated, n=3; VX-770 and VX-809-treated, n=3; untreated controls, n=3). The concentration levels were normalised to those of the untreated cells. **(D)** Formulations containing either 100 nM α ENaC siRNA (n=6) or control siRNA (n=3) were used in three sequential transfections of CFBE-BMI-1 cells grown at ALI for 4 weeks and the percentage of silencing was calculated 8 days after the third transfection. Silencing was normalised to the mean control siRNA set at 100%. The middle horizontal lines represent the median values. Asterisks indicate comparisons of specific formulations with statistical significance (*, $p<0.05$) determined by Mann-Whitney U non-parametric tests.

Figure 6 *In vivo* lung administration of siRNA-containing nanocomplexes. **(A)** Uptake of Dy677-siRNA formulations following oropharyngeal administration. 24 h later the mice were culled (n=3 per group) and organs (heart, lung, liver, kidneys, spleen and intestines) were extracted and imaged for fluorescence. **(B-D)** The remaining α ENaC mRNA was quantified by qRT-PCR in C57BL6 female mice after instillation of cationic nanocomplexes containing 16 μ g α ENaC siRNA (n=7) or control siRNA (n=6) at **(B)** 48 h and **(C)** 7 days. **(D)** The amount of remaining α ENaC mRNA detected by qRT-PCR in C57BL6 female mice at 72 h after the last of 3 instillations of cationic nanocomplexes containing 16 μ g α ENaC siRNA (n=8) or control siRNA (n=7). Silencing was normalised to the mean control siRNA set at 100%. Medians and IQRs are presented by horizontal lines. Asterisks indicate comparisons of specific formulations with statistical significance (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) determined by Mann-Whitney U non-parametric tests.

Figure 7 *In vivo* delivery of siRNA-containing nanocomplexes. Representative images of H&E stained murine lung sections following single **(A)** and triple **(B)** instillations of cationic α ENaC siRNA nanocomplexes (n=3 for both), or a triple instillation (n=3) of cationic control siRNA nanocomplexes **(C)**. All triple-instilled treated mice received 3 doses of nanocomplexes containing 16 μ g siRNA on alternate days and the lungs were harvested 48 h after the third instillation. The mice that had one instillation of nanocomplexes containing 16 μ g siRNA, also had their lungs harvested 48 h following instillation. Scale bars = 100 μ m.

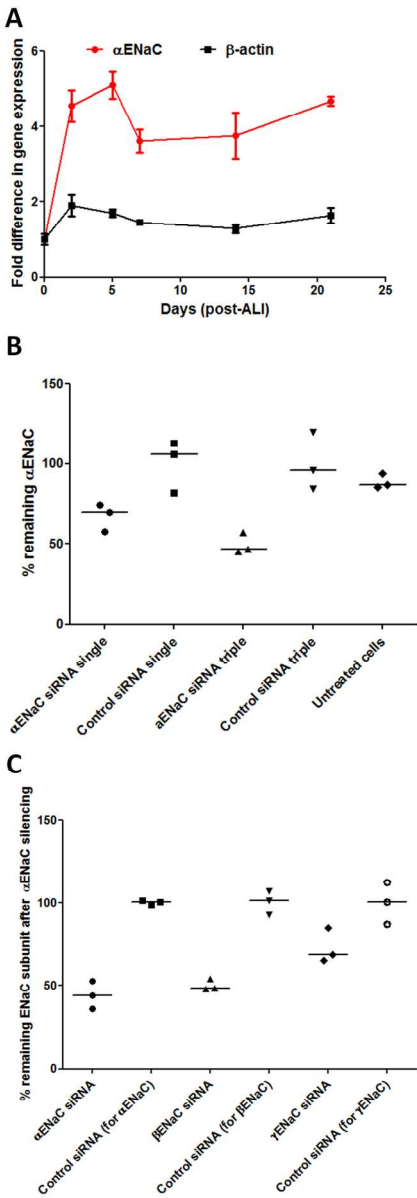
Particle	Diffusion in PGM ($\text{cm}^2 \text{s}^{-1}$)	D_m/D_w (PGM)	Diffusion in NM ($\text{cm}^2 \text{s}^{-1}$)	D_m/D_w (NM)	Diffusion in CFM ($\text{cm}^2 \text{s}^{-1}$)	D_m/D_w (CFM)
siRNA	3.75×10^{-9}	1.57×10^{-2}	1.31×10^{-8}	1.11×10^{-2}	2.33×10^{-9}	2.66×10^{-3}
RTN	1.38×10^{-8}	1.93×10^{-1}	8.71×10^{-9}	9.12×10^{-2}	2.67×10^{-9}	3.74×10^{-2}
PS	5.75×10^{-9}	4.82×10^{-2}	-	-	N/A	N/A

Table 1. A comparison of the nanoparticle effective diffusion rate in mucus (D_m) and the effective diffusion rate in water (D_w) of the receptor-targeted nanocomplex (RTN), polystyrene nanoparticles (PS) and siRNA alone through 3 different types of static layers of mucus. D_m/D_w is the relative restriction of diffusion in mucus compared to water. N/A= data not applicable as effective translocation was not achieved. PGM= pig gastric mucus, NM= normal human airway mucus and CFM= cystic fibrosis human airway mucus.



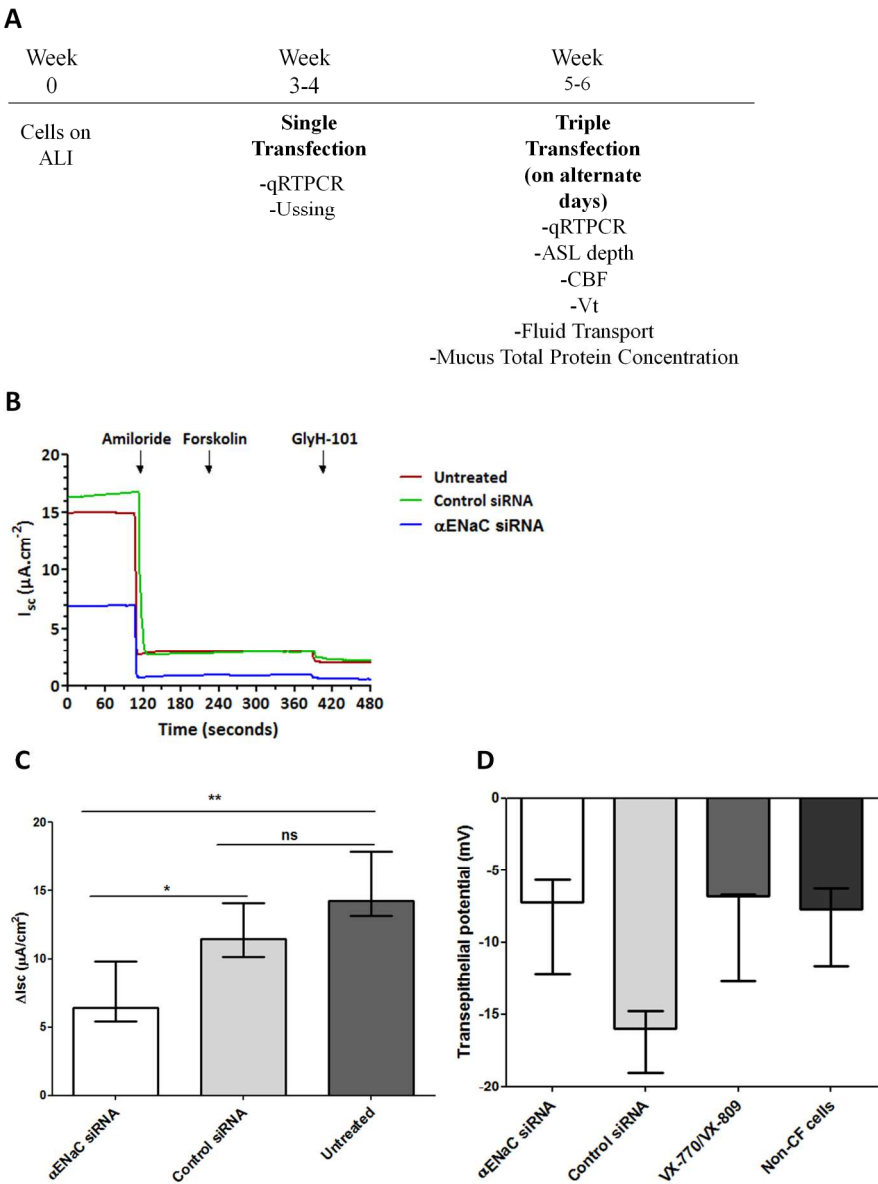
Nanoparticles translocate vertically through the mucus barrier.

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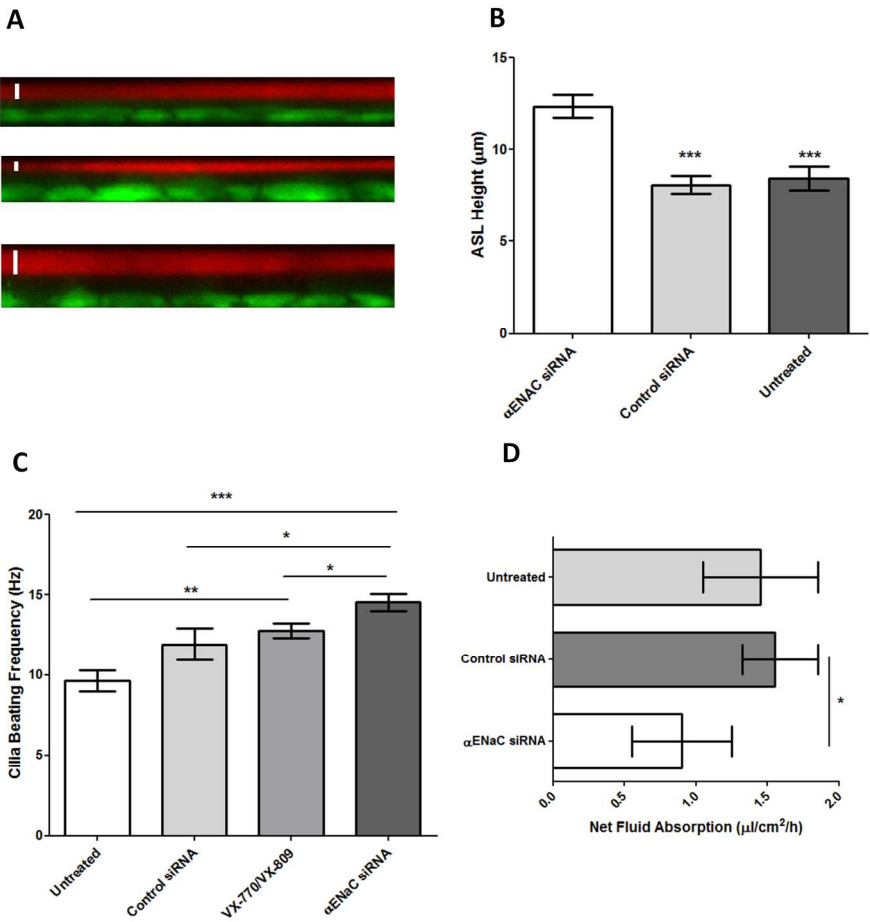
ENaC expression over time of primary cells growing at ALI and silencing of ENaC.

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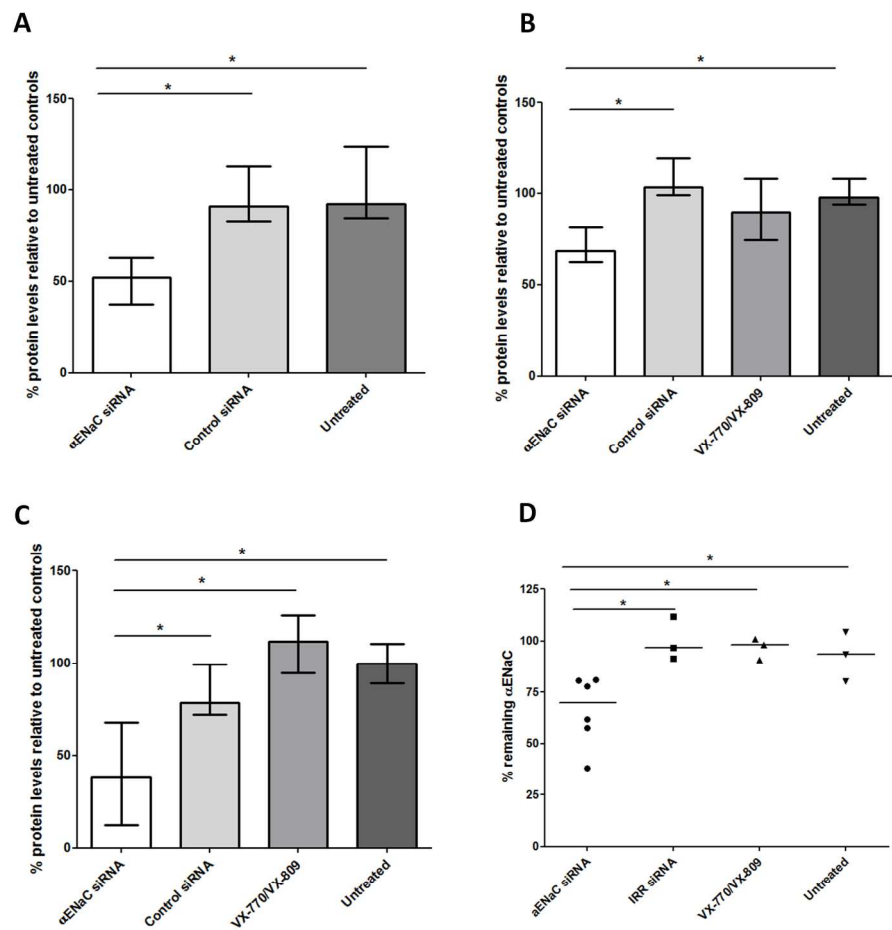
Single transfection of CFBE air-liquid interface cell monolayers with nanocomplexes containing αENaC siRNA reduces the amiloride-sensitive short circuit current (Isc).

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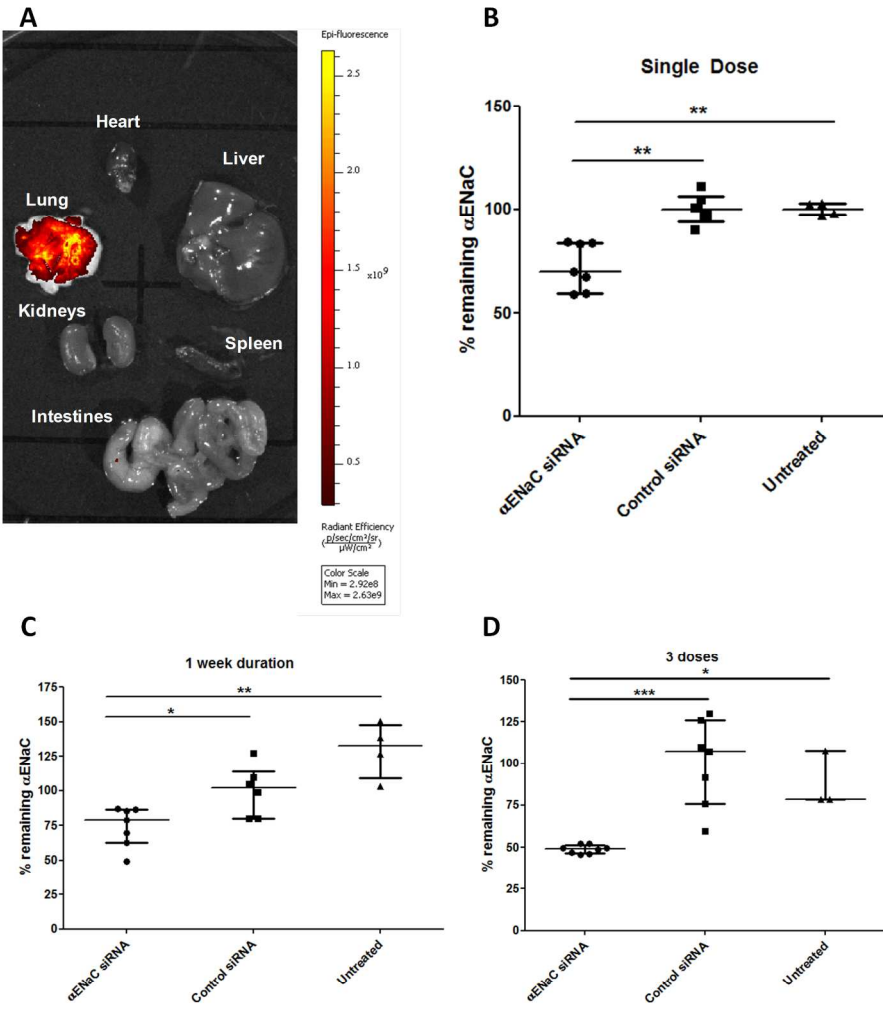
Effect of triple transfections of CFBE ALI cell with nanocomplexes containing αENaC siRNA.

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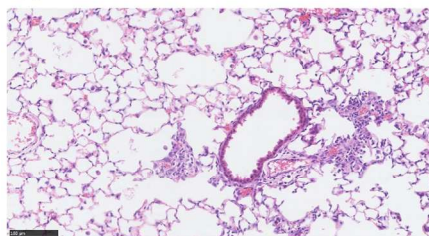
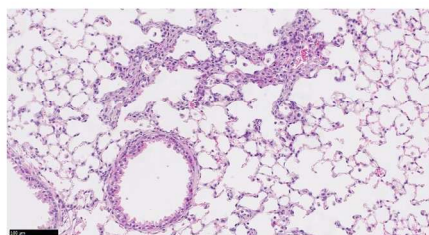
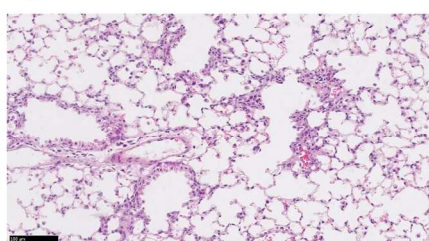
Protein concentration in mucus collected at different time points from transfected or untreated CFBE cells grown at ALI and in vitro silencing after 3 transfections.

254x338mm (300 x 300 DPI)



In vivo lung administration of siRNA-containing nanocomplexes.

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In vivo delivery of siRNA-containing nanocomplexes.

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Effective silencing of ENaC by siRNA delivered with epithelial-targeted nanocomplexes in human cystic fibrosis cells and in mouse lung

Aristides D. Tagalakis, Mustafa M. Munye, Rositsa Ivanova, Hanpeng Chen, Claire M. Smith, Ahmad M. Aldossary, Luca Z. Rosa, Dale Moulding, Josephine L. Barnes, Konstantinos N. Kafetzis, Stuart A. Jones, Deborah L. Baines, Guy W.J. Moss, Christopher O’Callaghan, Robin J. McNulty, and Stephen L. Hart

ONLINE DATA SUPPLEMENT

Supplemental Methods

Materials

1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The targeting peptide E (K16GACSERSMNFCG) was synthesised by China Peptides (Shanghai, China). Pig stomachs were purchased from Mutch Meats (Whitney, UK). The normal mucus from a healthy subject and the human mucus from a CF patient collected through the MucilAirTM system were bought from Epithelix (Epithelix Sarl, Geneva, Switzerland). Amine modified polystyrene nanoparticles, sodium chloride, ethylene diamine tetra acetic acid (EDTA) and phenylmethyl sulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (Dorset, UK). The α ENaC siRNA sequence (5'-GCAGUGAUGUUCCUGUUGA-3') was selected from an initial comparison of four candidate siRNAs in transfections of 16HBE14o- cells (as it gave the highest silencing) and were all obtained from Thermo Fisher Scientific (Northumberland, UK) for *in vitro* transfections. The α ENaC siRNA for *in vivo* administration (gene id: Scnn1a - AM16830) was obtained from Life Technologies (Paisley, UK). Non-targeting control siRNA was obtained from Thermo Fisher Scientific (Northumberland, UK) and from Life Technologies (Paisley, UK). Cy-3 Silencer GAPDH siRNA was bought from Thermo Fisher Scientific (Northumberland, UK) and used for the mucus translocation studies. VX-770 and VX-809 were bought from Generon (Maidenhead, UK). GlyH-101 (1) was bought from Merck Chemicals (Nottingham, UK) and Forskolin from Cambridge Bioscience (Cambridge, UK). All other chemicals were bought from Sigma-Aldrich (Dorset, UK).

Liposome and Nanocomplex Formation

Lipids were dissolved in chloroform at 10 mg/mL then a lipid film was produced in a rotary evaporator by slowly evaporating the chloroform. Lipids were rehydrated with sterile, distilled water whilst constantly rotated overnight, and then sonicated in a water bath to reduce their size. Cationic liposomes made were: DOTMA:DOPE at 1:1 molar ratio.

Cationic receptor-targeted nanocomplex (RTN) formulations (at a weight ratio of 1:4:1, liposome: peptide: DNA or siRNA) were made by first adding the peptide to the liposome, followed by addition of the siRNA (or DNA) with rapid mixing and incubation for 30 minutes at room temperature (RT) to allow for complex formation. Non-targeted RTNs were made in the same way.

Particle Sizing and Zeta Potential Measurements

Nanocomplex preparations were diluted with distilled water to a final volume of 1 mL at a concentration of 5 µg/mL with respect to DNA or siRNA. They were then analysed for size and charge (ζ potential) using a Malvern Nano ZS (Malvern, UK). The following specifications were used: automatic sampling time of 10 measurements/sample, refractive index of 1.330, dielectric constant 78.5, viscosity 0.8872 cP and temperature of 25°C. Dispersion Technology Software (DTS) version 5.03, which was provided by the manufacturer, was used for data processing.

Porcine Gastric Mucus Preparation

The stomachs of freshly slaughtered pigs were opened along their greater curvature, inverted, any food content removed mechanically. The stomachs were rinsed with double-distilled water

and the mucus lining was gently removed by scraping using a plastic spatula and transferred to a container containing a protease inhibiting buffer composed of 200 mM sodium chloride, 0.02 % (w/v) sodium azide, 5 mM EDTA and 1 mM PMSF. The mucus was mixed well with an equal volume of the protease inhibiting buffer and homogenised with a mixer for 2 min. The mucus-containing mixture was centrifuged at 11,200 g for 45 min at 4°C. The supernatant was poured into Visking dialysis tubing (MWCO: 12-14 kDa, Fisher Scientific, Loughborough, UK) and dialysed against deionised water for 24 h. The dialysed mucus solution was concentrated using an Amicon ultra-filtration stirred cell (Model 8400, 10 kDa membrane, Merck Millipore, UK) under nitrogen at a pressure of 40 psi and at 4°C. The retained mucus sample was collected into a bottle and frozen at -20°C until used.

Static Mucus Diffusion

Three different types of mucus were used: pig gastric mucus prepared in-house as above and non-CF and CF human mucus supplied commercially by Epithelix Sarl (Geneva, Switzerland). A 35 µm thick mucus barrier was established by application of 1 µl of pig gastric mucus, human CF or non-CF mucus to Transwell membranes (Costar, UK) and equilibrated at 37°C for 0.5 h. The mucus barrier thickness and confluence was confirmed using microscopy and the flux of sodium fluorescein demonstrated it was a rate limiting barrier. The translocation of the siRNA alone, the RTN and commercial cationic polystyrene nanoparticles was determined over time from an apical donor nanosuspension into an aqueous receiver fluid. 600 µL of Tris buffer pH 7.4 was used as the receiver fluid. 3 µL of nanoparticle suspensions containing Cy3-siRNA or siRNA alone at a concentration of 140 ng/µl were applied onto the surface of the barrier. At appropriate intervals, samples were removed from the receiver chamber and a 'fit for purpose'

(in terms of precision and limit of detection) fluorescence spectroscopy method was used for detection at a maximum excitation of 550 nm and emission of 570 nm (FLx800 Microplate Fluorescence Reader, Bio-TEK Instruments, Swindon, UK). The cumulative concentrations (ng/cm²) of translocated nanoparticles were quantified and plotted over time and the effective diffusion rates in mucus (D_m) using Fick's law

$$\frac{dM}{dt} = \frac{DC}{h}$$

where $\frac{dM}{dt}$ was the flux, D was the diffusion coefficient, C was the concentration of the permeant in the donor solution and h was the thickness of the barrier) and the effective diffusion rates in water (D_w) using Stokes' law

$$D = \frac{KT}{6\pi\eta r}$$

(where D is the diffusion coefficient, K is the Boltzman constant, T is the temperature, η is the viscosity and r is the particle radius) were also calculated.

Cell Culture and Transfections

The 16HBE14o- cells (kindly provided by Dieter Gruenert, California Pacific Medical Center Research Institute, San Francisco, CA, USA) were maintained in Minimum Essential Medium Eagle's modification (Sigma-Aldrich, Dorset, UK) at 37°C in a humidified atmosphere with 5% CO₂. Tissue culture medium was supplemented with 10% heat-inactivated foetal bovine serum (FBS, Life Technologies, Paisley, UK), 2 mM L-glutamine (Life Technologies) and 0.1 mM non-essential amino acids (Sigma-Aldrich, Dorset, UK). Cystic Fibrosis Bronchial Epithelial cells (primary CFBE) and Normal Human Bronchial Epithelial cells (primary NHBE) were

obtained from Epithelix (Epithelix SàRL, Genève, Switzerland). Primary cells were transduced in house (2) with a lentiviral vector expressing the anti-senescent *BMI-1* oncogene, obtaining CFBE-Bmi1 (CFBE) and NHBE-Bmi1 (NHBE) cells. Primary cells in submerged cultures were grown on 1% collagen-coated plastic flasks (PureCol® Bovine Collagen Solution, Type I, Advanced BioMatrix, San Diego, CA, USA) in Bronchial Epithelial Growth Medium (BEGM, Lonza) supplemented with Bovine Pituitary Extract, hydrocortisone, Human Epidermal Growth Factor, epinephrine, transferrin and insulin. Submerged cultures were transfected in 24 well plates (7×10^4 cells per well) and transfected with nanocomplexes prepared as described above and diluted with OptiMEM (Life Technology, Paisley, UK) at different siRNA concentrations in replicates of 3. Plates were centrifuged at 400g for 5 min and then incubated for 4 h at 37°C. The transfection solution was then removed and replaced with complete media. The plate was then incubated for 48 h to determine expression of the gene.

For ALI culture, primary cells were grown in 12 mm collagen-coated transwell or snapwell inserts (Polyester (PET) Membrane Transwell-Clear Inserts, Corning, Corning Inc. Life Sciences, Tewksbury, MA, USA) at a seeding density of 1.5×10^5 or 1×10^6 viable cells per insert. Two days after seeding on transwells or snapwells, the BEGM media was removed from the apical side of the insert and the basolateral media changed to BEGM ALI medium (1:1 DMEM-Hi glucose: BEGM containing supplements) supplemented with 100 nM retinoic acid (Sigma-Aldrich, Dorset, UK). Medium was changed 3 times per week and mucus produced on the apical surface was removed once per week by gentle washing with PBS. Cells were transfected in snapwells with siRNA nanocomplexes at 100 nM final concentration (diluted with OptiMEM) by applying 1000 μ l to the basolateral surface and 125 μ l to the apical surface (no mucus was removed prior to transfection) and incubated for 4 hours at 37°C. The transfection

solution was then removed and replaced with ALI media. The plate was then incubated for 48 h or longer to determine gene expression or to perform functional studies. Transepithelial electrical resistance (R_t) of CFBE monolayers grown at ALI was measured at regular intervals using the EVOM Voltohmmeter (WPI, Hitchin, UK).

For certain transfections, 48h after the transfection or at different time points the apical surface was washed with 75 μ l of PBS to collect the mucus. The amount of protein present in each mucus sample was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hemel Hempstead, UK) in a FLUOstar Optima luminometer (BMG Labtech, Aylesbury, UK).

Ussing Chamber Studies

Confluent CFBE monolayers were grown on snapwell clear membrane supports (Corning, UK) at ALI for 3 weeks and treated with or without nanoparticles. 48 h after treatment, they were then mounted into Ussing chambers and bathed from both sides with physiological saline containing (in mM) NaCl 117, NaHCO₃ 25, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2 and D-glucose 11 (pH 7.4). This was maintained at 37°C and gassed with a premixed gas (21% O₂ + 5% CO₂). Monolayers were maintained under open-circuit conditions and the spontaneous transepithelial potential (V_t) was monitored until a stable value was reached (~15 min) using a DVC 4000 voltage/current clamp and recorded via a PowerLab computer interface. Drugs were added in the order of amiloride (10 μ M, apical), forskolin (25 μ M, apical and basolateral) and GlyH-101 (10 μ M, apical). NHBE cells were used as controls.

ASL Depth Measurement

CFBE cells grown in snapwells in ALI were transfected 3 times (every other day) with nanocomplexes containing siRNA to ENaC (n=4) or control siRNA (n=4) or were left untreated as controls (n=4). ASL depth (i.e. depth of both the mucus and PCL layers) was measured 72 h after the last transfection using confocal microscopy. The ASL in each snapwell was washed twice with PBS and then labeled with 20 μ l PBS containing 2 mg/ml Rhodamine B isothiocyanate-dextran (10 kDa; Sigma-Aldrich, Dorset, UK) by apical application the day prior to the experiment. The following day the CFBE cells were stained using 5 μ M Calcein-AM (Thermo Fisher Scientific, Northumberland, UK) dissolved in culture medium for at least 60 min and introduced to the basolateral compartment of the insert. Perfluorocarbon (300 μ l; Sigma-Aldrich, Dorset, UK) was added to the apical compartment of the insert in order to prevent ASL evaporation. Fluorescent images of the epithelial layer and ASL height were obtained using a confocal microscope (Zeiss AxioObserver LSM 710 40x/NA1.2 plan-apochromat water objective, Jena, Germany). Samples were imaged in a #1.5 glass bottom dish (FD35, Fluorodish, World Precision Instruments, Hitchin, UK). Z stack acquisition used the optimal z-step as calculated in the Zen Software. For each snapwell, 5 different microscope fields randomly chosen were XZ scanned. Images were analysed with ImageJ/Fiji, using a macro to resliced images in an XZ orientation and threshold the ASL stain to produce a binary image for accurate manual height measurements. In each microscope field, the ASL height was measured in 9 separate regions randomly determined over the surface of the monolayer and then averaged.

Transepithelial Potential

Cystic fibrosis bronchial epithelial cells (CFBE), cultured in snapwells at ALI, were left untransfected or transfected 3 times (every other day) with nanocomplexes containing siRNA to

ENaC or control siRNA and then placed in an environmental chamber. The transepithelial potential (V_t) of these samples was measured with the pipette immersed in the ASL using a Scanning Ion Conductance Microscope (SICM; OpenIOLabs, Cambridge, UK) paired with an Axopatch 200B amplifier (Molecular Devices, CA, USA). V_t measurements were conducted using the amplifier in current clamp mode ($I=0$ mV). V_t was defined as the electrical potential between a high-resistance borosilicate pipette ($R>80$ M Ω) immersed directly into the ASL, and an Ag/AgCl bead electrode immersed in Ringer's solution on the basolateral side. In certain wells, the CFTR corrector VX-809 (10 μ M) was added on the basolateral surface 1 day prior to ASL depth measurement, followed by addition of the potentiator VX-770 (10 μ M) during measurement (again on the basolateral surface for approximately 20 min).

Transepithelial Fluid Transport Measurement

To quantify fluid absorption, CF cells were cultured in ALI as explained above. Following three sequential transfections (on alternate days) with ENaC or control siRNA, the apical surface of the cells was washed 48 h after the last transfection with 500 μ l of a saline solution at RT containing (in mM): 137 NaCl, 2.7 KCl, 8.1 Na₂HPO₄, 1.5 KH₂PO₄, 1 CaCl₂, 0.5 MgCl₂. After washing, the apical side of the epithelium was covered with 150 μ l of the same solution and 200 μ l of mineral oil to prevent evaporation (3) and the cells returned to the incubator. After 24 h, the apical fluid was carefully removed, centrifuged to separate the mineral oil, and the volume of aqueous phase measured. The net flux across the epithelium is calculated as $J= (V_i-V_f)/A_t$, where V_i and V_f are the initial and final apical volumes, A is the = epithelium area, and t is the time interval between addition of V_i and recovering of remaining fluid V_f .

Assessment of Ciliary Beating

Respiratory primary CFBE cell cultures grown in snapwells in ALI were transfected 3 times (every other day) with nanocomplexes containing siRNA (α ENaC or control) or treated with VX-770 and VX-809 as above or left untreated. The cultures were not washed for the duration of the experiment. 5 days after the last transfection they were placed in an incubation chamber (37°C, 5% CO₂) and were observed via an inverted microscope system (Nikon, UK) equipped with an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu, Japan). For each experimental condition, readings of ciliary beat frequency (CBF) were calculated from ten ciliated areas in the snapwell using ciliaFA software as previously described (4, 5).

Western Blot

Transfections for protein extraction were performed in a 6-well plate. The protein extraction procedure was performed on ice. Media was aspirated and cells were washed 3 times with ice-cold PBS. 30 μ L of RIPA buffer supplemented with PMSF (Invitrogen, Paisley, UK) and protease inhibitor cocktail (Invitrogen, Paisley, UK) were added per well and incubated for 1 h on ice. Cells were then scraped and transferred in a 1.5 mL Eppendorf tube and kept on ice for 30 min vortexing every 10 minutes. Cells were then centrifuged at 14,000xg for 10 min at 4°C and the supernatants collected (cell lysates). The amount of protein in the lysates was measured with the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Northumberland, UK) reading the absorbance at 595nm in a FLUOstar Optima luminometer (BMG Labtech, Aylesbury, UK).

Approximately 30 μ g of the protein lysate were denatured at 95 °C for 5 min and loaded onto a 4-12% NuPAGE Bis-Tris pre-cast polyacrylamide gel. The gel was run at 100V for 15

min and 150V for 1 h in 1X NuPage running buffer. Proteins were transferred from the gel to a nitrocellulose membrane at 25V for 2.5 h in 1X NuPage transfer buffer (Life Technologies, Paisley, UK). The membrane was rinsed in di-ionised H₂O), washed 3x for 5 min in Tris-buffered saline/Tween (TBS-T) and subsequently incubated in a blocking buffer containing 5% milk powder in TBS-T for 1 h at RT. The membrane was then incubated with primary antibody in blocking buffer at 4 °C overnight. The primary antibodies used were polyclonal rabbit anti-αENaC (PAI-920; Thermo Fisher Scientific, Northumberland, UK) at 1:750 dilution and mouse monoclonal anti-β-actin (AC-15; Sigma-Aldrich, Dorset, UK) at 1:5000 dilution. The membranes were then washed 3x for 5 min in TBS-T and incubated in secondary antibody in blocking buffer at RT for 1h. The secondary antibody for αENaC was polyclonal swine anti-rabbit HRP (horseradish peroxidase) at 1:1700 dilution (Dako, Ely, UK) and for β-actin polyclonal goat anti-mouse HRP at 1:1000 dilution (Dako, Ely, UK). Then the membranes were washed 3x for 5 min in TBS-T and developed with Clarity Western ECL substrate (1:1 of peroxidase and luminol/enhancer reagent; Bio-Rad, Hemel Hempstead, UK). The western blot densities were analysed using ImageJ software (NIH, Bethesda, USA).

In Vivo Delivery and Histology of Lung Tissues

Female C57Bl6 mice 6-8 week old were purchased from Charles River (Margate, UK). All procedures were approved by UCL animal care policies and were carried out under Home Office Licenses issued in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (UK). Cationic nanocomplexes were prepared as described above at a final siRNA concentration of 0.29 mg/mL. Mice were instilled oropharyngeally following gaseous isoflurane induced anaesthesia (6, 7) with nanocomplexes in 55 µL (made in 5% glucose, v/v) containing

16 μ g siRNA (α ENaC or control siRNA), with untreated mice used as controls. In biodistribution studies, mice were instilled oropharyngeally as above with nanocomplexes in 55 μ L (made in 5% glucose, v/v) containing 16 μ g of siRNA-Dy677. Experiments were performed with replicates of 3 mice. 24 hours after injection, the mice were culled and organs (lung, liver, heart, kidneys, intestines and spleen) were resected and imaged using an IVIS Lumina Series III imaging system (PerkinElmer, Seer Green, UK). The images were processed using the Living Image software (PerkinElmer, Seer Green, UK).

48 h or 7 days post-administration and the lungs excised and snap-frozen in liquid nitrogen and stored at -80°C till needed. In other studies siRNA-containing nanocomplexes were instilled 3 times (every other day) and the lungs excised 72 h after the 3rd instillation.

For the histology studies, mice were culled 48 h following oropharyngeal instillation of nanocomplexes and the lungs inflation fixed *in situ* with 4% paraformaldehyde at a pressure of 20 cm. The lungs were placed in 4% (w/v) PFA for 3 h followed by overnight incubation in 15% (w/v) sucrose/PBS and then a brief wash in 50% (v/v) ethanol and stored in 70% (v/v) prior to processing to paraffin wax.

RNA Extraction From Cells and Mouse Tissues

After 48 h, submerged cells were trypsinised and homogenised with Qiagen shredders (Qiagen, Crawley, UK). Cells grown on ALI were washed in PBS, scraped, centrifuged at 14,000g for 5 min and homogenised with Qiagen shredders. Total RNA was extracted from the homogenate using the RNeasy Kit (Qiagen, Crawley, UK), following the manufacturer's protocol and each sample underwent DNase treatment (Invitrogen, Paisley, UK) to eliminate any potential genomic DNA contamination. The samples were stored at -80 °C.

Mouse lungs were homogenised with the Precellys24 tissue homogenizer (2 cycles x 5600 rpm, 30 sec per cycle). Total RNA was extracted from mouse lysates using the RNeasy kit according to the manufacturer's instructions (Qiagen, Crawley, UK). RNA was checked for integrity using the Agilent 2100 Bioanalyzer (Wokingham, UK). All RNA samples had a RNA integrity number (RIN) of more than 9 indicating high quality RNA. Each sample underwent DNase treatment as above.

Quantitative Real-time PCR (qRT-PCR)

Total RNA (200 ng per reaction) was used in a one-step qRT-PCR (SensiFast Probe Hi-Rox One-Step Kit; Bioline, London, UK) that combines the reverse transcription with the quantitative PCR reaction. Human (Hs00168906_m1) or mouse (Mm01182998_g1) αENaC, human (Hs99999903_m1) or mouse β-actin (Mm00607939_s1), human βENaC (Hs01548617_m1) and human γENaC (Hs00168918_m1) were quantified by Taqman primers and probes (Thermo Fisher Scientific, Northumberland, UK). The qRT-PCR assays were performed in a Bio-Rad CFX96 Real-Time PCR Detection System with the following parameters: 45°C for 20 min, 95°C for 2 min and then 40 cycles at 95°C for 15 sec and 60°C for 1 min. Relative expression levels were calculated using the delta-delta Ct ($2^{-\Delta\Delta C_t}$) method (8).

Supplemental Figure Legends

Figure S1. Silencing efficiency of 16HBE14o- cells transfected with cationic ENaC siRNA nanocomplexes. **(A)** Representative western blot of α ENaC protein from 16HBE14o- cells transfected with 75 nM of siRNA-containing nanocomplexes (n=3). α ENaC major forms of 90 kDa and 65 kDa bands are shown in the blot. Untr= untreated cells. **(B)** The quantification by densitometry analysis of the % of remaining α ENaC protein of the above blot is shown for the 65 kDa protein band of each group.

Figure S2. Expression of the different ENaC subunits in primary CFBE cells grown at ALI. CFBE cells were grown at ALI for 4 weeks and were then harvested and qRTPCR was performed in order to determine the expression of the different ENaC subunits. α ENaC and β ENaC are expressed relative to γ ENaC which is set at 1 (n=3). The middle horizontal lines represent the median values while the upper and lower horizontal bars represent the IQR. Asterisks indicate comparisons of specific formulations with statistical significance (***, $p < 0.001$; Mann-Whitney non-parametric tests were performed).

Figure S3. Representative I_{sc} traces from untreated NHBE monolayers in Ussing chambers. This figure was used in work we conducted simultaneously and has been very recently published. (2)

Figure S4. Transepithelial electrical resistance (R_t) measurements on transfected and non-transfected CFBE-Bmi1 monolayers. The monolayers were cultured in snapwells and transfected with 100 nM of α ENaC siRNA or control siRNA or were left untreated. 48 h later their R_t was

measured. The middle horizontal lines represent the median values while the upper and lower horizontal bars represent the IQR (n=3-7). There was no statistical difference between the groups tested (Mann-Whitney non-parametric tests were performed).

Figure S5. Radiant efficiencies (photons s⁻¹ cm⁻² steradian⁻¹ per μW cm⁻²) of organs/tissues following oropharyngeal administration of nanocomplexes carrying Dy677-siRNA. 24 h later the mice were culled (n=3 per group) and organs (heart, lung, liver, kidneys, spleen and intestines) were extracted and imaged for fluorescence with the IVIS III system. The uptake of siRNA-Dy677 was significantly more in the lungs when compared to the intestines, the latter being the only other organ where fluorescence was detected. *, *p*<0.05 (Mann-Whitney non-parametric tests were performed to compare uptake differences between organs). Radiant efficiencies were measured using a Living Image 4.0 software package.

Figure S6. Weights of mice (in grams) **(A)** before and 7 days after a single instillation of nanocomplexes containing 16 μg siRNA and **(B)** during 3 instillations. Mann-Whitney non-parametric tests were performed. Each individual mouse is represented by a symbol. The lines are the means of the weights for each group of mice.

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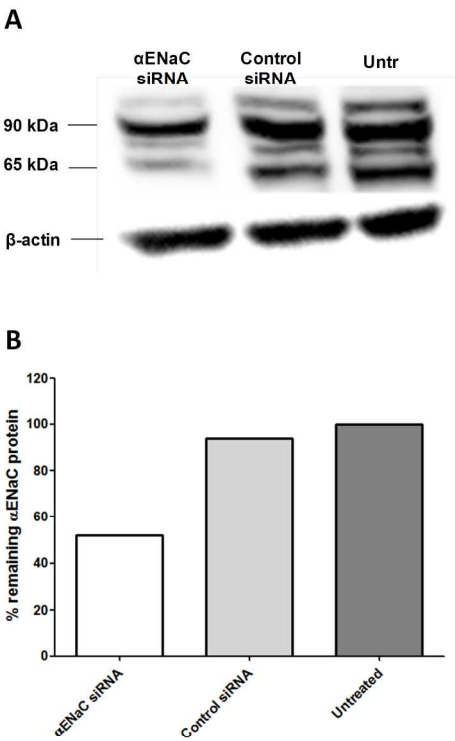


Figure S1

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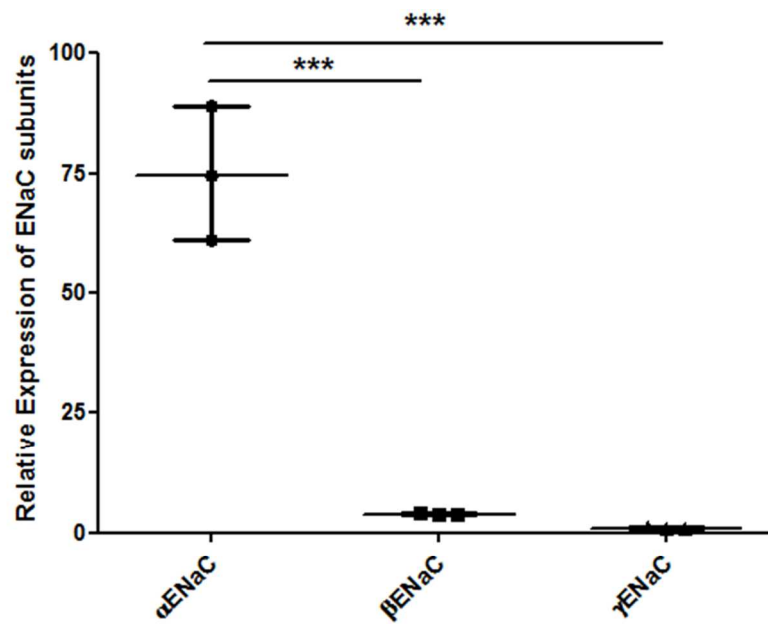


Figure S2

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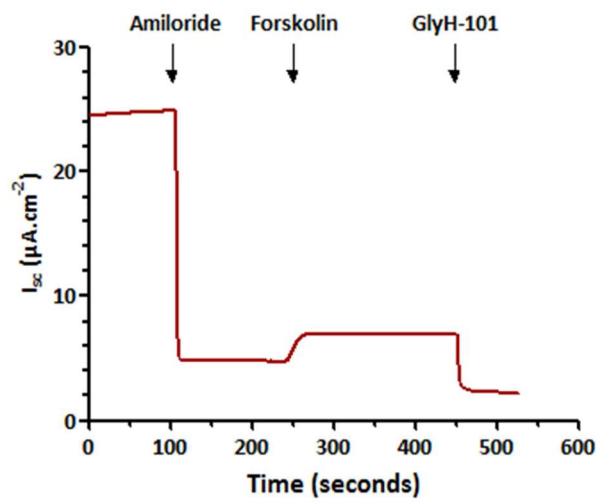


Figure S3

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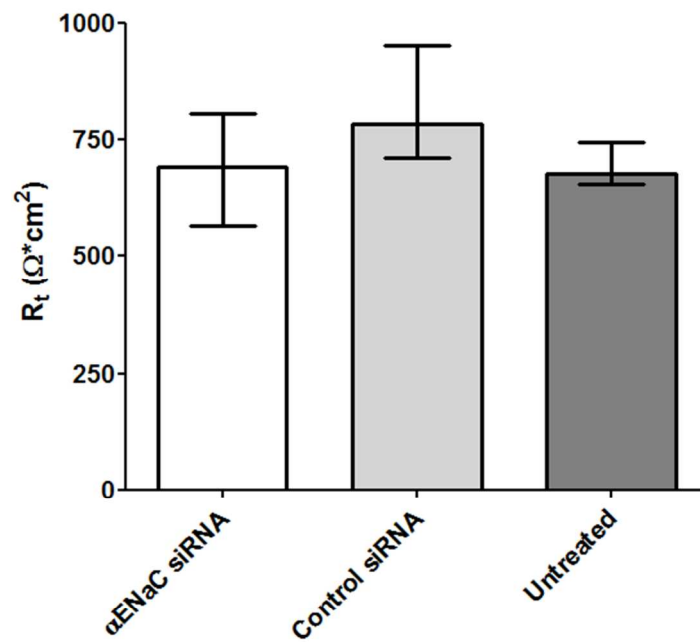


Figure S4

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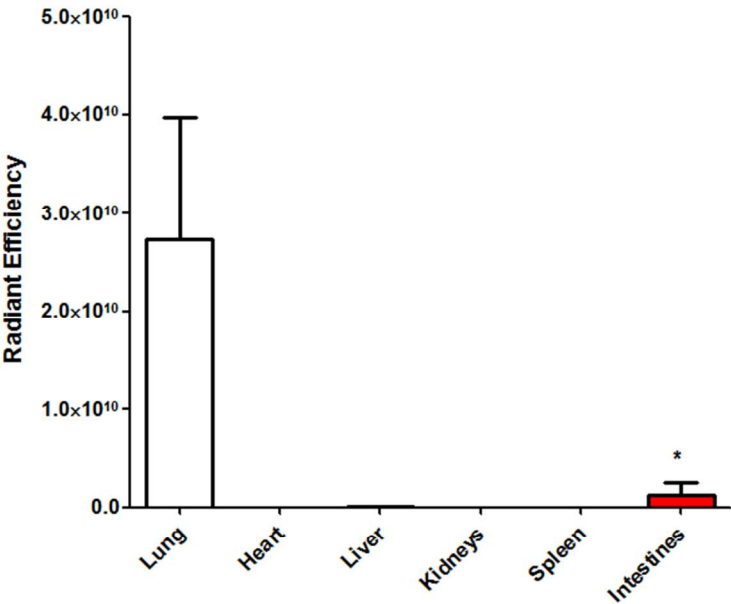


Figure S5

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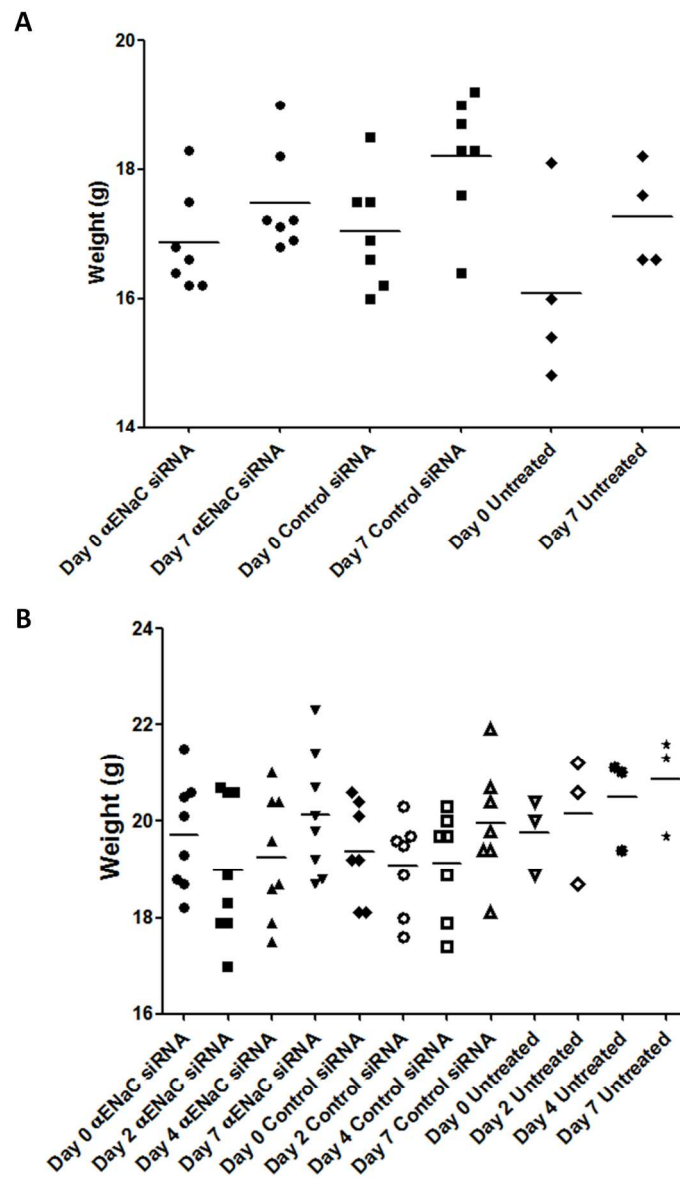


Figure S6

254x338mm (300 x 300 DPI)

Formulation	Normal human mucus (ng/cm ²)	Cystic Fibrosis mucus (ng/cm ²)	Pig Gastric mucus (ng/cm ²)
RTN	961.5 ± 44.8	441.1 ± 52.7	1046.1 ± 47.6
siRNA	917.3 ± 60.1	390.1 ± 15.8	472.0 ± 8.8
Polystyrene beads	not done	100.0 ± 57.6	782.7 ± 123.0

Table S1. Nanoparticle translocation through mucus. Cumulative concentrations through different mucus sources of the receptor-targeted nanocomplexes (RTN), siRNA and polystyrene nanoparticles (PS) were determined from the accumulated fluorescence over a period of 1 hour. Values are averages ± SEM.

αENaC siRNA dose	αENaC silencing %	βENaC silencing %	γENaC silencing %
Single	30	51	0
Triple	54	not done	not done

Table S2. *In vitro* siRNA silencing of ENaC in epithelial cells. CFBE cells grown at ALI were transfected with RTN formulations containing either αENaC siRNA or control siRNA at 100 nM. The percentage of silencing of α, β and γ ENaC subunits was calculated 48 h after transfection (n=3 per formulation). Values shown are the differences of the median silencing of ENaC siRNA treated group compared to their respective control siRNA group.

αENaC siRNA dose	αENaC	Control siRNA	Untreated
Amiloride-sensitive (<i>I</i> _{sc}) μA/cm ²	6.4 (IQR: 5.4-9.8)	11.5 (IQR: 10.1-14.1)	14.3 (IQR: 13.2-17.9)
<i>R</i> _t Ω cm ²	693.5 (IQR: 565.7-805.8)	783.2 (IQR: 711.1-953.2)	676.7 (IQR: 653.1-744.2)

Table S3. The effects of siRNA transfection on amiloride-sensitive short circuit current *I*_{sc} and transepithelial electrical resistance (*R*_t). Values are medians with the Interquartile range (IQR).

Analysis	α ENaC siRNA	Control siRNA	VX-770/ VX-809 CFBE	NHBE untreated	CFBE untreated
Transepithelial potential (V_t), mV	-7.2 (IQR: -5.7 to -12.2)	-16.0 (IQR: -14.8 to -19.0)	-6.8 (IQR: -6.7 to -12.7)	-7.7 (IQR: -6.3 to -11.6)	not done
ASL depth, μm	12.1 (IQR: 10.7-14.9)	7.9 (IQR: 6.4-9.8)	not done	not done	8.2 (IQR: 5.8-11.1)
Ciliary beat frequency, Hz	14.5 \pm 0.5	11.9 \pm 1.0	12.8 \pm 0.5	not done	9.6 \pm 0.7
Net fluid absorption, μl/cm²/h	0.9 (IQR: 0.6-1.3)	1.6 (IQR: 1.3-1.9)	not done	not done	1.5 (IQR: 1.1-1.9)

Table S4. Effects of triple-dose α ENaC silencing on transepithelial potential (V_t), airway surface liquid depth (ASL), ciliary beat frequency and net fluid absorption. Values are shown as mean \pm SEM for ciliary beat frequencies. All other values are medians with the Interquartile range (IQR).

siRNA dose	% α ENaC silencing 48h-72h	% α ENaC silencing, 1 week after administration
Single	30	23
Triple	58	not done

Table S5. *In vivo* silencing of α ENaC. Following α ENaC siRNA administration, silencing was determined by qRT-PCR at 48 h and 1 week. Triple dosing was also performed and the percentage of silencing was calculated 72 after the final dose. Values shown are the differences of the median silencing of α ENaC siRNA treated group compared to their respective control siRNA group.



The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny¹, William J Browne², Innes C Cuthill³, Michael Emerson⁴ and Douglas G Altman⁵

¹The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, ²School of Veterinary Science, University of Bristol, Bristol, UK, ³School of Biological Sciences, University of Bristol, Bristol, UK, ⁴National Heart and Lung Institute, Imperial College London, UK, ⁵Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

ITEM		RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Page 1
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Page 3
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Page 5-6 Page 6, paragraph 2
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	P. 5, para 2
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Supplementary file, page 11
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	P.28, Sections 1 and 2
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Supp. P. 11- P.12
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Supp.P. 11

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	NA
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	P. 28 sections 1 and 2
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	NA
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	P.16, para 2 "body weight"
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	P. 7
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	NA
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50% ²). b. If any animals or data were not included in the analysis, explain why.	P. 28, sections 1 and 2
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	P.28, sections 1 and 2
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	NA
DISCUSSION			
Interpretation/scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results ² . c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	P.13-P17
Generalisability/translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	P.17
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	P. 2

References:

- Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
- Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332. <https://mc.manuscriptcentral.com/thorax>